Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;37:193-200
DOI: 10.1159/000430344
Published online: August 20, 2015
Accepted: July 07, 2015

1421-9778/15/0371-0193$39.50/0

Original Paper

DCP-LA Activates Cytosolic PKCε by Interacting with the Phosphatidylserine Binding/Associating Sites Arg50 and Ile89 in the C2-Like Domain

Takeshi Kanno[a] Ayako Tsuchiya[a] Tadashi Shimizu[b] Miyuki Mabuchi[b]
Akito Tanaka[b] Tomoyuki Nishizaki[a]

[a]Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, Nishinomiya, [b]Laboratory of Chemical Biology, Advanced Medicinal Research Center, Hyogo University of Health Sciences, Kobe, Japan

Key Words
DCP-LA • PKCε • C2-like domain • Phosphatidylserine binding/associating sites • Direct interaction

Abstract
Background/Aims: The linoleic acid derivative DCP-LA selectively and directly activates PKCε. The present study aimed at understanding the mechanism of DCP-LA-induced PKCε activation. ***Methods:*** Point mutation in the C2-like domain on PKCε was carried out, and each kinase activity was monitored in PC-12 cells using a föerster resonance energy transfer (FRET) probe with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) at the N- and C-terminal ends of PKCε, respectively, or in the cell-free systems using a reversed phase high-performance liquid chromatography (HPLC). Intracellular PKCε mobilization was monitored in PC-12 cells using mRuby-conjugated PKCε. DCP-LA binding to PKCε was assayed using a fluorescein conjugated to DCP-LA at the carboxyl-terminal end (Fluo-DCP). Uptake of DCP-LA into cells was measured in PC-12 ells. ***Results:*** In the FRET analysis, DCP-LA decreased the ratio of YFP signal intensity/CFP signal intensity in PC-12 cells and in the cell-free kinase assay, DCP-LA increased area of phosphorylated PKC substrate peptide, indicating DCP-LA-induced PKCε activation. These effects were significantly suppressed by replacing Arg50 and Ile89 by Ala or Asn in the C2-like domain of PKCε. In the fluorescent cytochemistry, DCP-LA did not affect intracellular PKCε distribution. In the cell-free binding assay, Fluo-DCP, that had no effect on the potential for PKCε activation, bound to PKCε, and the binding was inhibited only by mutating Ile89. Extracellularly applied DCP-LA was taken up into cells in a concentration-dependent manner. Although no activation was obtained in the cell-free kinase assay, the broad PKC activator PMA activated PKCε in PC-12 cells in association with translocation towards the cell surface, which was inhibited by mutating I89A. ***Conclusion:*** Unlike PMA DCP-LA activates cytosolic PKCε by binding to the phosphatidylserine binding/associating sites Arg50 and Ile89, possibly at the carboxyl-terminal end and the cyclopropane rings, respectively.

Prof. Tomoyuki Nishizaki
Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, (Japan)
Tel. +81-798-45-6397, Fax +81-798-45-6649, E-Mail tomoyuki@hyo-med.ac.jp

Introduction

Accumulating evidence has shown that the linoleic acid (LA) derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]octanoic acid (DCP-LA) could improve dementia including Alzheimer's disease [1-8]. The effect is mainly attributed to selective and direct activation of PKCε involving delivery of α7 ACh receptor to the presynaptic membrane surface and release of neurotransmitters [9-12]. In our earlier study, DCP-LA-induced PKCε activation was inhibited by adding phosphatidylserine (PS) [13]. This raises the possibility that DCP-LA activates PKCε by interacting with the PS binding site on PKCε. The accurate binding sites, however, have not been identified.

The present study was conducted to answer this issue. We show here that unlike the broad PKC activator phorbol 12-myristate 13-acetate (PMA) DCP-LA binds to Arg50 and Ile89 in the C2-like domain of PKCε and activates PKCε localized in the cytosol, but not at the plasma membrane. We also show that LA activates PKCε by binding to the same sites as DCP-LA.

Materials and Methods

DNA subcloning and site-directed mutagenesis

The plasmid containing the normal rat PKCε was constructed. For the PKCε mutants the amino acid residues in the C2-like domain of PKCε were replaced by Ala or Asn.

FRET analysis

The vector for FRET probe with CFP and YFP at the N- and C-terminal ends of PKCε, respectively, was constructed and transfected into PC-12 cells using a Lipofectamine LTX-PLUS (Invitrogen, Carlsbad, CA, USA). Forty-eight hours later FRET monitoring was carried out with Zeiss LSM510 META inverted microscope (Oberkochen, Germany) by the method as described previously [14]. The CFP and YFT fluorescent signals were detected at an absorbance of 474 and 506 nm, respectively, using an excitation light of 458 nm. The FRET ratio (YFP signal intensity/CFP signal intensity) was calculated using an ImageJ software (National Institutes of Health, USA).

Fluorescent cytochemistry

PKCε, conjugated with the red fluorescent protein eqFP611, mRuby, was expressed into PC-12 cells, and intracellular PKCε mobilization was monitored with a confocal scanning laser microscope (Axiovert/LSM510; Carl Zeiss, Oberkochen, Germany).

Cell-free PKCε assay

In the cell-free systems, PKCε activity was assayed by the method previously described [13, 15]. Briefly, a synthetic PKC substrate peptide (10 μM) was reacted with wild-type and mutant PKCε in a Ca^{2+}- and PS-free solution containing DCP-LA, lipids or PMA at 30°C for 5 min. After loading on a reversed phase HPLC (LC-10ATvp, Shimadzu Co., Kyoto, Japan), a substrate peptide peak and a new product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured, and phosphorylated substrate peptide (pmol/min) was used as an index of PKC activity. PKCε activities induced by DCP-LA, lipids and PMA were calculated by subtracting the basal PKCε activities in the presence of DMSO (Δpmol/min).

PKCε binding assay

Fluo-DCP was constructed by the method previously described [16]. Briefly, wild-type and mutant PKCε were reacted with Fluo-DCP in the presence and absence of non-conjugated DCP-LA, and then, separated by blue native-polyacrylamide gel electrophoresis. The fluorescent signals were visualized using a FluoroPhoreStar3000 (Anatech, Tokyo, Japan).

Assay of intracellular distribution of DCP-LA

After treatment with DCP-LA for 5 min, lysates from PC-12 cells were separated into the cytosolic and plasma membrane components. DCP-LA in each component was labeled with 9-anthryldiazomethane (ADAM)(Funakoshi, Tokyo, Japan) and then, the sample solution was loaded onto the HPLC system. ADAM was detected at an excitation wavelength of 365 nm and an emission wavelength of 412 nm using a fluorescence detector.

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferroni correction.

Results

DCP-LA activates PKCε without translocation towards the cell surface

We initially monitored PKCε activity in PC-12 cells using a FRET probe, which has CFP and YFP at the N- and C-terminal ends of PKCε, respectively (Fig. 1A). A conformational change of PKCε from the inactive form to the active form leads to an enhancement of the CFP signal intensity from the YFP signal intensity (Fig. 1A), and therefore, a decrease and an increase in the ratio (YFP signal intensity/CFP signal intensity) correspond to activation and inactivation of PKCε, respectively (Fig. 1B). PMA, DCP-LA and LA significantly decreased the FRET ratio as compared with that for DMSO (Fig. 1C). This implies that PMA, DCP-LA and LA activate PKCε in PC-12 cells.

PKCε contains the PS binding/associating sites in the C2-like domain (Fig. 1D) [17]. PMA-induced PKCε activation was significantly inhibited by replacing Trp23 (W23A), Arg50 (R50A) and Ile89 by Ala (I89A) (Fig. 1E). Similarly, activation of mutant PKCεW23A (mPKCεW23A), -R50A and -I89A induced by DCP-LA was significantly attenuated as compared with that for wild-type PKCε (wPKCε) (Fig. 1E). These results suggest that PMA and DCP-LA activate PKCε by interacting with the PS binding/associating sites Trp23, Arg50 and Ile89 in the C2-like domain.

In the fluorescent cytochemistry in PC-12 cells expressing mRuby-conjugated PKCε, PMA translocated wPKCε to the cell surface, but otherwise such effect was not found with DCP-LA and LA (Fig. 1F). This suggests that PKCε activation does not parallel with PKCε translocation towards the cell surface. PMA did not affect intracellular mPKCεI89A distribution, while mPKCεW23A, -R50A and -Y91A were translocated towards the cell surface in a fashion similar to that for wPKCε (Fig. 1F).

DCP-LA activates PKCε by interacting with Arg50 and Ile89 in the C2-like domain

In the cell-free PKCε assay, DCP-LA-induced activation of mPKCεR50A, -I89A and -I89N (replacement of Ile89 by Asn) significantly reduced as compared with that for wPKCε, but no significant reduction was found with mPKCεW23A and other mPKCε with replacement of Arg26 (R26A), Arg32 (R32A), His85 (H85A), Asp86 (D86A), Pro88 (P88A), Gly90 (G90A), Tyr91 (Y91A), Asp92 (D92A) and Asp93 (D93A) by Ala (Fig. 2A).

Likewise, LA activated wPKCε to an extent similar to that for DCP-LA, and the activation was significantly reduced by mutating Arg50 and Ile89 (Fig. 2B). Another unsaturated free fatty acid oleic acid (OA) and the saturated free fatty acid stearic acid (SA) activated wPKCε, but to a lesser extent than that for DCP-LA or LA, and the activation was also reduced by mutating Arg50 and Ile89 (Fig. 2C, D). Surprisingly, no activation of wPKCε was obtained with PMA (Fig. 2E). This, in the light of the fact that cell-free PKCε assay was carried out in a PS-free solution, suggests that PS is required for PMA-induced PKCε activation. Dioleoyl-PS (DO-PS) still activated wPKCε, and the activation was significantly reduced by mutating Ile89 and Gly90, but not Arg50 (Fig. 2F). This suggests that PS binds to Ile89 and Gly90, thereby partially activating PKCε.

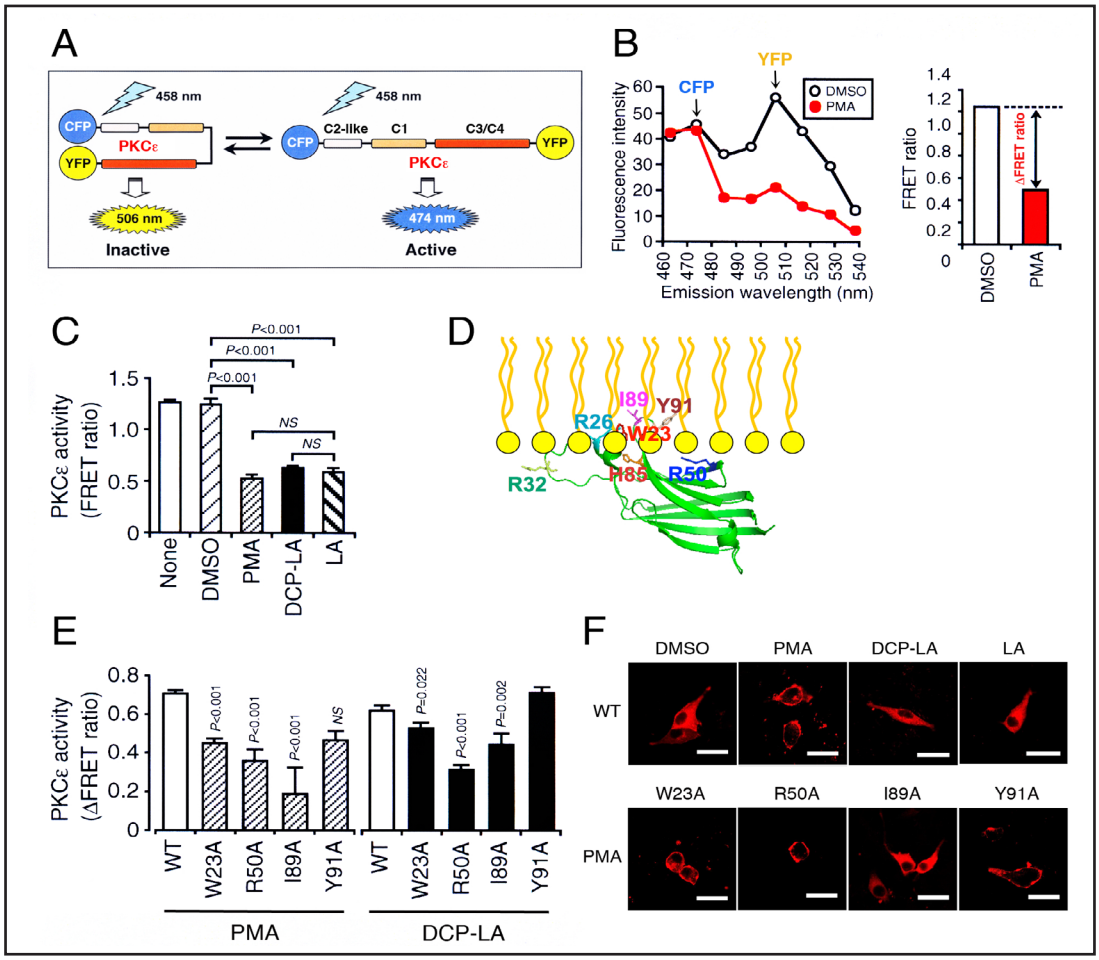


Fig. 1. FRET analysis. (A) A schema of the FRET probe. (B) The relation between the fluorescent intensities and absorbance wavelengths (left panel) and the FRET ratio (YFP signal intensity detected at an absorbance of 506 nm/CFP signal intensity detected at an absorbance of 474 nm). (C) The YFP and CFP signals were monitored in PC-12 cells expressing the FRET probe 15 min after treatment with DMSO, PMA (100 nM), DCP-LA (100 nM) or LA (100 nM). Data represent the mean (± SEM) FRET ratio (YFP signal intensity/CFP signal intensity)(n=10 independent experiments). *P* values, ANOVA followed by a Bonferroni correction. *NS*, not significant. (D) The PS binding/associating sites in the C2-like domain of PKCε. (E) Data represent the mean (± SEM) activities of wild-type (WT) and mutant PKCε (ΔFRET ratio)(n=10 independent experiments). *P* values as compared with wPKCε activity, ANOVA followed by a Bonferonni correction. (F) Fluorescent cytochemistry in PC-12 cells expressing mRuby 15 min after treatment with DMSO, PMA (100 nM), DCP-LA (100 nM) or LA (100 nM). Scale bars, 20 μm. Note that similar results were obtained from 10 independent experiments.

DCP-LA directly binds to free PKCε

To obtain evidence for DCP-LA binding to PKCε, we constructed Fluo-DCP (Fig. 3A). Fluo-DCP produced a single fluorescent signal band at 87 kDa in the electrophoresed gel, corresponding to the molecule of PKCε, and the signal was attenuated or abolished by co-treatment or pretreatment with non-conjugated DCP-LA (Fig. 3A). The signal intensity for Fluo-DCP binding to mPKCεI89A and -I89N was significantly lower than that to wPKCε, and no significant effect was obtained with the other mutants including mPKCεR50A (Fig. 3B). In the cell-free kinase assay, Fluo-DCP activated wPKCε to an extent similar to that for DCP-LA (Fig. 3C). These results indicate that DCP-LA directly binds to Ile89 in the PS binding site, to

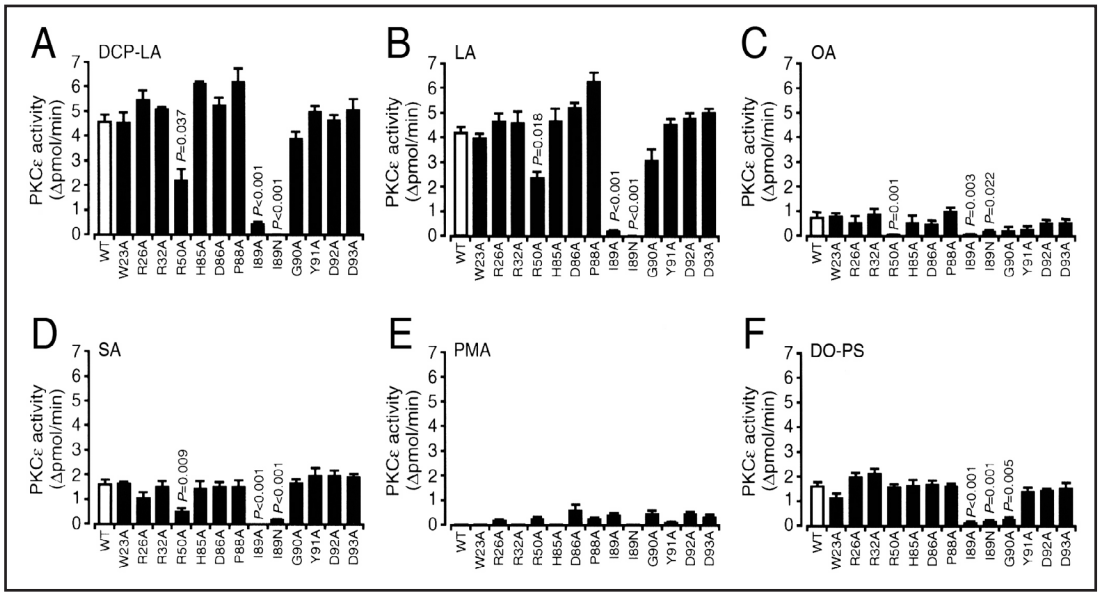


Fig. 2. Cell-free PKCε assay. Synthetic PKC substrate peptide (10 μM) was reacted with wild-type (WT) and mutant PKCε (0.1 μg/mL) as indicated in the absence and presence of DCP-LA (100 μM)(A), LA (100 μM)(B), OA (100 μM)(C), SA (100 μM)(D), PMA (1 μM)(E) or DO-PS (100 μM)(F) for 5 min. Data represent the mean (± SEM) PKCε activity (Δpmol/min)(n=4 independent experiments). *P* values as compared with WT PKCε activity, ANOVA followed by a Bonferonni correction.

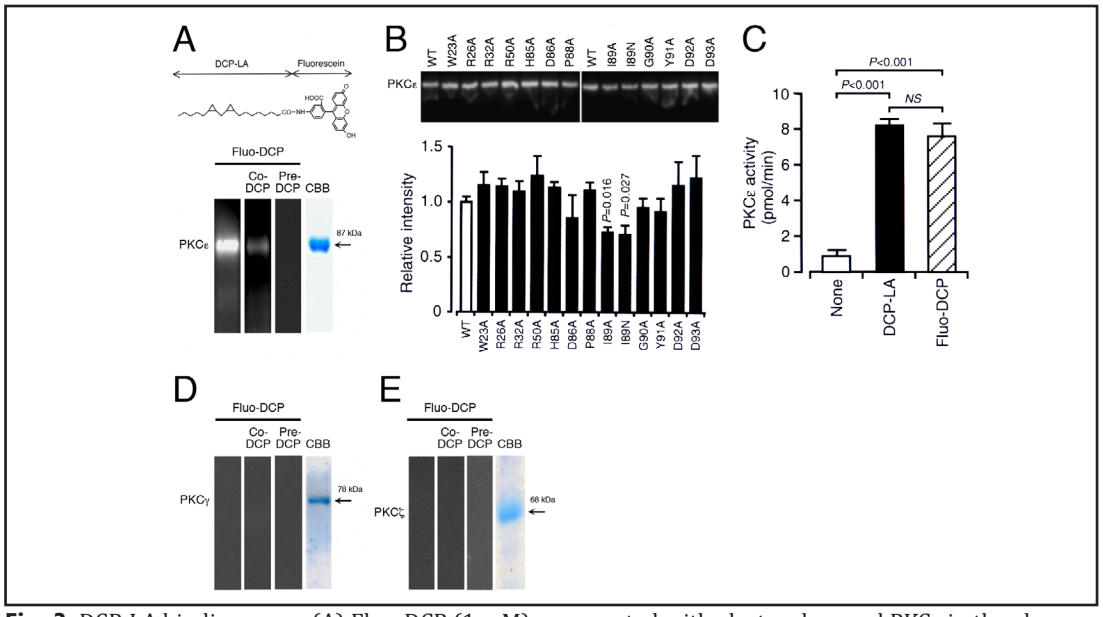


Fig. 3. DCP-LA binding assay. (A) Fluo-DCP (1 mM) was reacted with electrophoresed PKCε in the absence and presence of non-conjugated DCP-LA (Co-DCP)(1 mM) or after pretreatment with non-conjugated DCP-LA (Pre-DCP)(1 mM). CBB, Coomassie brilliant blue staining. Note that similar results were obtained with 4 independent experiments. (B) Fluo-DCP (1 mM) was reacted with electrophoresed wild-type (WT) and mutant PKCε as indicated. Data represent the mean (± SEM) signal intensity relative to that for wPKCε (n=4 independent experiments). *P* values as compared with the signal intensity for wPKCε, ANOVA followed by a Bonferonni correction. (C) PKCε activities induced by DMSO (None), DCP-LA (100 μM) and Fluo-DCP (100 μM). Data represent the mean (± SEM) PKCε activity (pmol/min)(n=4 independent experiments). *P* values, ANOVA followed by a Bonferonni correction. Fluo-DCP (1 mM) was reacted with electrophoresed PKCγ (D) or PKCς (E) in the absence and presence of non-conjugated DCP-LA (Co-DCP)(1 mM) or after pretreatment with non-conjugated DCP-LA (Pre-DCP)(1 mM).

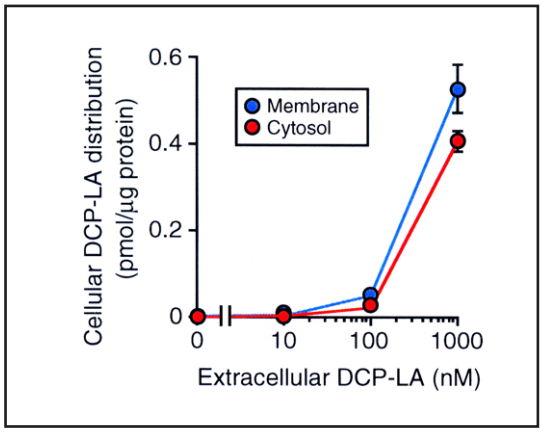


Fig. 4. Uptake of DCP-LA into cells. PC-12 cells were treated with DCP-LA at concentrations as indicated, and DCP-LA in the cytosolic and plasma membrane components was quantified with the HPLC systems. Data represent the mean (± SEM) DCP-LA concentration (pmol/μg protein)(n=4 independent experiments).

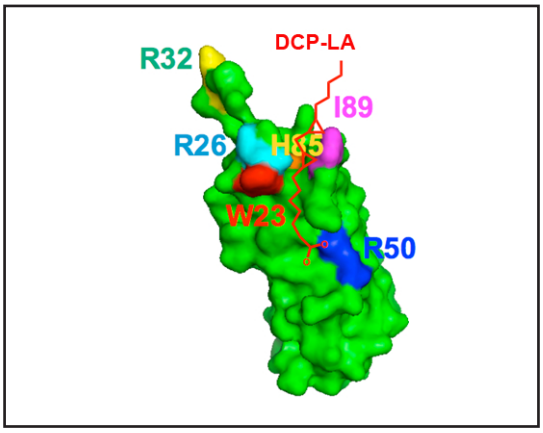
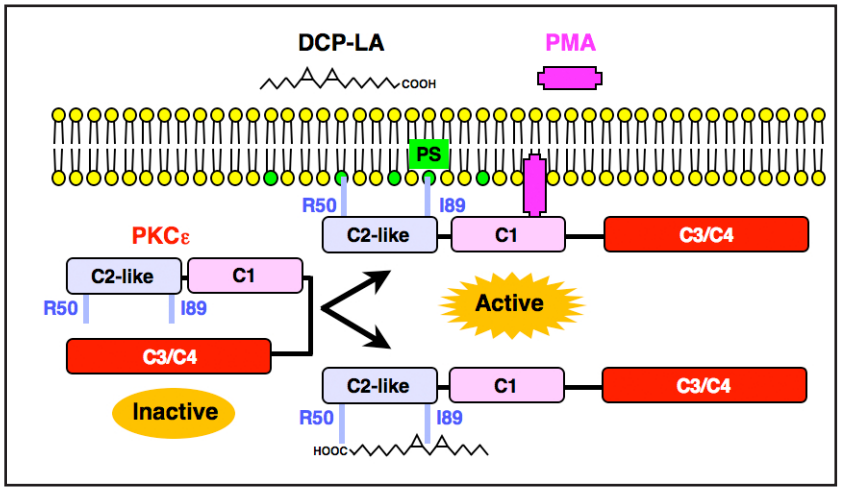


Fig. 5. Putative DCP-LA binding site in the C2-like domain of PKCε.

Fig. 6. A schematic diagram for DCP-LA- and PMA-induced PKCε activation.



activate PKCε. Fluo-DCP, on the other hand, produced no signal band binding to the classical PKC isozyme PKCγ and the atypical PKC isozyme PKCς (Fig. 3 D, E). Collectively, these results interpret that DCP-LA directly and preferentially binds to PKCε.

DCP-LA is taken up into cells

We finally examined whether extracellularly applied DCP-LA is taken up into cells. When extracellularly applied to PC-12 cells, cytosolic DCP-LA increased in a concentration (10 nM-1 μM)-dependent manner in parallel with increased concentrations in the plasma membrane (Fig. 4). This suggests that DCP-LA is capable of activating PKCε localized in the cytosol.

Discussion

PMA is shown to activate PKCε by binding to the C1 domain [18]. In the present study, PMA activated wPKCε in PC-12 cells, and the activation was inhibited by replacing Trp23,

Arg50 and Ile89 by Ala, yet no activation of wPKCε was obtained with PMA in the cell-free kinase assay using a PS-free solution. PMA translocated wPKCε towards the cell surface in PC-12 cells and the effect was cancelled in mPKCεI89A. These results, in the light of the finding that PS partially activates PKCε by interacting with Ile89 and Gly90, raise the possibility that an inactive form of PKCε binds to PS in the plasma membrane at Ile89, to make PKCε an open frame, allowing PMA to bind to the C1 domain and to activate PKCε fully; in other words, PS is indispensable for PMA-induced PKCε activation. Extracellularly applied PMA, therefore, would be trapped in the plasma membrane, where PMA binds to the C1 domain of PKCε linked by PS (Fig. 6). Likewise, diacylglycerol, which is produced by phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate *in vivo*, may also activate PKCε at the plasma membrane.

Unlike PMA DCP-LA activated PKCε in PC-12 cells without translocation towards the cell surface. This suggests that DCP-LA activates PKCε localized in the cytosol. In support of this notion, DCP-LA was actually taken up into cells. DCP-LA-induced activation of PKCε in PC-12 cells and under the cell-free conditions was suppressed by replacing Arg50 and Ile89 by Ala or Asn. This implies that DCP-LA activates PKCε by interacting with Arg50 and Ile89. Intriguingly, Fluo-DCP binding to PKCε was attenuated only by mutating Ile89, but not Arg50, although there was no significant difference in the potential of wPKCε activation between Fluo-DCP and DCP-LA. This, in the light of the fact that fluorescein is conjugated to DCP-LA at the carboxyl-terminal end, suggests that the carboxyl-terminal end on DCP-LA recognizes and binds to Arg50 on PKCε. Overall, the results presented here lead to a conclusion that extracellularly applied DCP-LA is taken up into cells, where DCP-LA activates PKCε by binding to Arg50 and Ile89 in the C2-like domain, possibly at the carboxyl-terminal end and the cyclopropane rings, respectively (Fig. 5, 6).

Like DCP-LA LA activated PKCε without translocation, and the activation was also inhibited by mutating Arg50 and Ile89. This suggests that LA activates PKCε by binding to Arg50 and Ile89 in the C2-like domain, possibly at the carboxyl-terminal end and the *cis*-double bonds, respectively. The results of the present study, thus, may represent novel insight into the mechanism of PKCε activation induced by *cis*-unsaturated free fatty acids as well as DCP-LA.

Acknowledgments

We thank H. Nagaya (Laboratory of Cell and Gene Therapy, Institute for Advanced Medical Sciences, Hyogo College of Medicine) for helping construction of the PKCε FRET probe vector.

Disclosure Statement

The authors declare no competing financial interests.

References

1 Tanaka A, Nishizaki T: The newly synthesized linoleic acid derivative FR236924 induces a long-lasting facilitation of hippocampal neurotransmission by targeting nicotinic acetylcholine receptors. Bioorg Med Chem Lett 2003;13:1037-1040.

2 Yamamoto S, Kanno T, Nagata, T, Yaguchi T, Tanaka A, Nishizaki T: The linoleic acid derivative FR236924 facilitates hippocampal synaptic transmission by enhancing activity of presynaptic α7 acetylcholine receptors on the glutamatergic terminals. Neuroscience 2005;130:207-213.

3 Nagata T, Yamamoto S, Yaguchi T, Iso H, Tanaka A, Nishizaki T: The newly synthesized linoleic acid derivative DCP-LA ameliorates memory deficits in animal models treated with amyloid-β peptide and scopolamine. Psychogeriatrics 205;5:122-126.
4 Yaguchi T, Nagata T, Mukasa T, Fujikawa H, Yamamoto H, Yamamoto S, Iso H, Tanaka A, Nishizaki T: Linoleic acid derivative DCP-LA improves learning impairment in SAMP8. Neuroreport 2006;17:105-108.
5 Yaguchi T, Fujikawa H, Nishizaki T: Linoleic acid derivative DCP-LA protects neurons from oxidative stress-induced apoptosis by inhibiting caspase-3/-9 activation. Neurochem Res 2010;35:712-717.
6 Nagata T, Tomiyama T, Mori H, Yaguchi T, Nishizaki T: DCP-LA neutralizes mutant amyloid β peptide-induced impairment of long-term potentiation and spatial learning. Behav Brain Res 2010;206:151-154.
7 Kanno T, Yaguchi T, Shimizu T, Tanaka A, Nishizaki T: 8-[2-(2-Pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid and its diastereomers improve age-related cognitive deterioration. Lipids 2012;47:687-695.
8 Nishizaki T, Kanno T, Gotoh, A: A newly synthesized linoleic acid derivative DCP-LA as a promising anti-dementia drug. Personalized Med Univ 2014;3:28-34.
9 Kanno T, Yaguchi T, Yamamoto S, Yamamoto H, Fujikawa H, Nagata T, Tanaka A, Nishizaki T: 8-[2-(2-Pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid stimulates GABA release from interneurons projecting to CA1 pyramidal neurons in the rat hippocampus via pre-synaptic α7 acetylcholine receptors. J Neurochem 2005;95:695-702.
10 Shimizu T, Kanno T, Tanaka A, Nishizaki T: α,β-DCP-LA selectively activates PKC-ε and stimulates neurotransmitter release with the highest potency among 4 diastereomers. Cell Physiol Biochem 2011;27:149-158.
11 Kanno T, Tanaka A, Nishizaki T: Linoleic acid derivative DCP-LA stimulates vesicular transport of α7 ACh receptors towards surface membrane. Cell Physiol Biochem 2012;30:75-82.
12 Kanno T, Tsuchiya A, Tanaka A, Nishizaki T: The linoleic acid derivative DCP-LA increases membrane surface localization of the α7 ACh receptor in a protein 4.1N-dependent manner. Biochem J 2013;450:303-309.
13 Kanno T, Yamamoto H, Yaguchi T, Hi R, Mukasa T, Fujikawa H, Nagata T, Yamamoto S, Tanaka A, Nishizaki, T: The linoleic acid derivative DCP-LA selectively activates PKC-ε, possibly binding to the phosphatidylserine binding site. J Lipid Res 2006:47:1146-1156.
14 Violin JD, Zhang J, Tsien RY, Newton AC. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J Cell Biol 2003;161:899-909.
15 Kanno T, Tsuchiya A, Shimizu T, Nakao S, Tanaka A, Nishizaki T: Effects of newly synthesized DCP-LA-phospholipids on protein kinase C and protein phosphatases. Cell Physiol Biochem 2013;31:555-564.
16 Tsuchiya A, Kanno T, Nagaya H, Shimizu T, Tanaka A, Nishizaki T: PTP1B inhibition causes Rac1 activation by enhancing receptor tyrosine kinase signaling. Cell Physiol Biochem 2014;33:1097-1105.
17 Ochoa WF, Garcia-Garcia J, Fita I, Corbalan-Garcia S, Verdaguer N, Gomez-Fernandez JC: Structure of the C2 domain from novel protein kinase Cε. A membrane binding model for Ca^{2+}-independent C2 domains. J Mol Biol 2011;311:837-849.
18 Farah CA, Nagakura I, Weatherill D, Fan X, Sossin WS: Physiological role for phosphatidic acid in the translocation of the novel protein kinase C Apl II in Aplysia neurons. Mol Cell Biol 2008;28:4719-4733.